AD_____

Award Number DAMD17-98-1-8046

TITLE: Identification of the Receptor of the WNT-1 Signaling Molecule

PRINCIPAL INVESTIGATOR: Raymond Habas, Ph.D.
Xi He, Ph.D.

CONTRACTING ORGANIZATION: Children's Hospital
Boston, Massachusetts 02115

REPORT DATE: May 1999

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 1999	Annual Summary (6 Apr 98 - 5 Apr 99)

4. TITLE AND SUBTITLE
Identification of the Receptor of the WNT-1 Signaling Molecule

5. FUNDING NUMBERS
DAMD17-98-1-8046

6. AUTHOR(S)
Raymond Habas, Ph.D.
Xi He, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Children's Hospital
Boston, Massachusetts 02115

E*Mail: habas_r@hub.tch.harvard.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The Wnt gene family encodes secreted signaling molecules that play important roles in mammary tumorigenesis and embryonic development . Wnt-1, the founding member, was identified as an oncogene that upon ectopic expression resulted in mice mammary tumors. Delineation of the mechanisms of Wnt-1 signaling can provide insights into the molecular nature of mammary tumor formation.

Recent studies suggest that the *frizzled* family of proteins may encode the receptors for the Wnt proteins and it is likely that a *frizzled* protein may encode for the receptor for Wnt-1. This hypothesis was tested using the *Xenopus* axis duplication system using a co-injection strategy with pools of *fz* molecules and a Wnt1-CD8 molecule. The ability of the *fz* molecules in one of the pools to mediate the secondary axis formation of Wnt1-CD8 has lead to the identification of two *fz* proteins which may potentially serve as the Wnt-1 receptor. Further experiments are in progress to verify these findings and experiments to identify molecules that interact with the cytoplasmic portion of the fz receptor are being attempted.

The elucidation of the receptor and downstream effectors for the Wnt-1 signaling molecule can provide valuable insights into how this ligand functions in mammary tumor formation.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

__✓__ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

__✓__ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Raymond Hahn 99/4/30

PI - Signature Date

TABLE OF CONTENTS

Introduction

The Wnt gene family encodes secreted signaling molecules that play important roles in mammary tumorigenesis and embryonic development. Wnt-1, the founding member of the Wnt gene family, was identified as an oncogene that upon ectopic expression resulted in mice mammary tumors. Delineation of the mechanisms of Wnt-1 signaling can provide insights into the molecular nature of mammary tumor formation. Recent studies suggest that the *frizzled* (*fz*) family of proteins may encode the receptors for the Wnt proteins. It was demonstrated that the *Drosophila* Wnt homologue, *wingless* (*wg*), was only able to bind to the surface of cells transfected with the *fz* related protein, dFz2. Studies in *Xenopus* embryos have identified a *fz* protein, hFz5, as the receptor for the Wnt-5A ligand. Based on these findings it is therefore likely that member of the *fz* family encodes for the receptor for the Wnt-1 molecule. This hypothesis can be tested using the established *Xenopus* axis duplication system. The identification and elucidation of the receptor for the Wnt-1 signaling molecule can provide valuable insights into how this ligand functions in mammary tumor formation.

BODY

Major Project covering Task 1 in Statement of Work (Months 1-14)

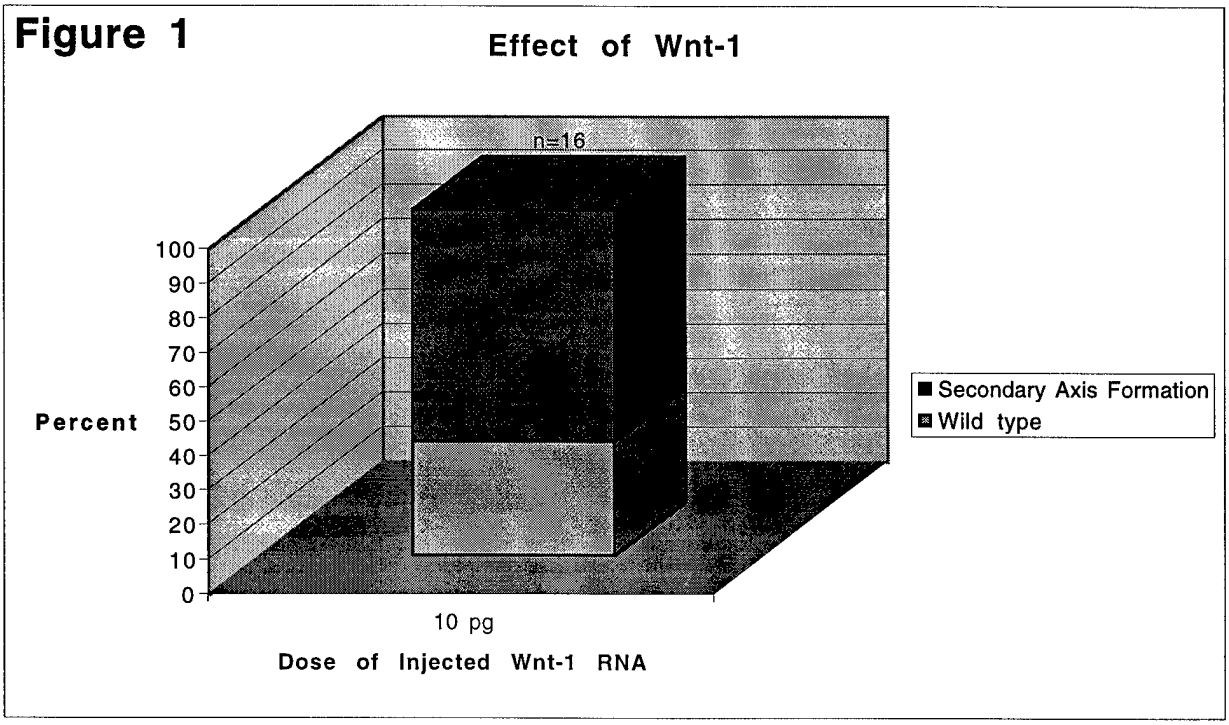
The *Xenopus* axis duplication has proven to be a useful biological assay for Wnt function as demonstrated by the elucidation of a *fz* family member, hFz5, as the receptor for Wnt-5A (He et al., 1997). In this assay, injection of Wnt-5a does not induce axis duplication and strictly relies on co-injected hFz5, but not other *fz* proteins, to achieve this. I have attempted to use this approach to identify the receptor(s) for Wnt-1.

There is one important modification of this approach required as Wnt-1 has been shown to directly induce secondary axis duplication without any co-injected *fz* molecules (Parkin et al., 1993). Perhaps Wnt-1 achieves this by activation of an endogenous *fz* protein(s) already present in the embryo. The modification involved reducing the quantity of injected Wnt-1 mRNA to suboptimal concentrations where minimal or no secondary axis formation is observed. Such a modification has been tested using the *Drosophila* homologue, *wg*, which like Wnt-1 can induce secondary axis duplication (Dr. Xi He, unpublished results). In these experiments, the amount of injected *wg* RNA was reduced to suboptimal levels so that *wg* could only inefficiently induce secondary axis duplication. Coinjection of *wg* with the RNA for dFz2, a known receptor for *wg*, was seen to significantly enhanced axis duplication by the suboptimal concentrations of *wg* RNA, whereas the same amount of dFz2 RNA alone did not induce secondary axis duplication. This result may be explained by the fact that the ligand-receptor complex formation between the reduced concentration of *wg* protein and an endogenous *fz* protein(s) is suboptimal. However

increasing the protein concentration of dFz2 from injected RNA permits effective *wg*-dFz2 complex formation and leads to enhanced signaling. By the same principle, this modified approach should be applicable to Wnt-1 and its receptor(s).

In order to determine whether there was a concentration of Wnt-1 that can provide a suboptimal concentration measured by its inability to induce secondary axis in Xenopus embryos, a concentration of Wnt-1 RNA of 10 pg per embryo was injected. However this concentration was induced secondary axis formation in greater than 80% of the embryos injected (Figure 1). These results are similar to the previous published data of Parkin et al., 1993. These results demonstrated a complication as further lower doses of injections could not attempted due to the inability to accurately measure and ensure accuracy of the injected RNA.

Figure 1

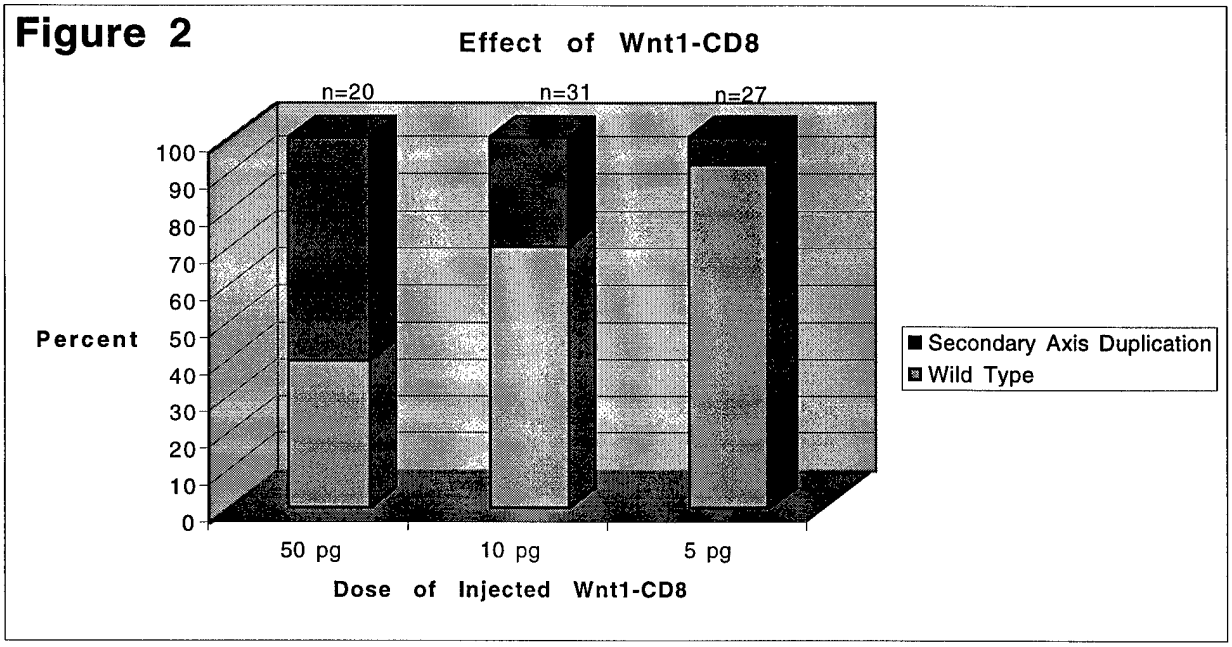


To bypass this complication a modified Wnt-1 construct was utilized. This construct contains the Wnt-1 coding sequence fused in frame with the protein CD8 (Parkin et al., 1993). The rational for the use of this construct was twofold. Firstly it has been demonstrated that this construct can induce secondary axis duplication but it requires a much higher quantity of injected RNA to achieve this outcome. Almost a twenty-five fold higher concentration of the Wnt1-CD8 RNA was required to achieve the same effect observed with Wnt-1 RNA (Parkin et al., 1993). This lowered activity of the Wnt1-CD8 fusion can therefore allow for a better discrimination of the range of RNA that would provide a suboptimal concentration. Also because the Wnt1-CD8 fusion protein will be tethered to the plasma membrane of cells expressing this construct, this

may allow for a more efficient co-localization and interaction of Wnt molecule with its putative injected frizzled molecule.

To delineate the range of Wnt1-CD8 mRNA that can provide a suboptimal concentration and not induce secondary axis duplication, injections were performed into Xenopus embryos. A range of 5 pg to 50 pg was tested. As figure 2 displays, the Wnt-1-CD8 construct was much weaker in its ability to induce secondary axis formation. This effect was dose dependent and 10 pg was observed to be an excellent suboptimal dose. The secondary axis inducing properties of 10 pg of Wnt1-CD8 was observed to be around 30% which provides a good baseline for measuring any increase or decrease in this observed phenotype in the presence of a *fz* protein. The possibility that a *fz* protein may have repressive effects on the axis inducing properties of the Wnt1-CD8 construct could not be ruled out. As such a baseline of 30% which can detect an increase or decrease in this phenotype was chosen.

Figure 2

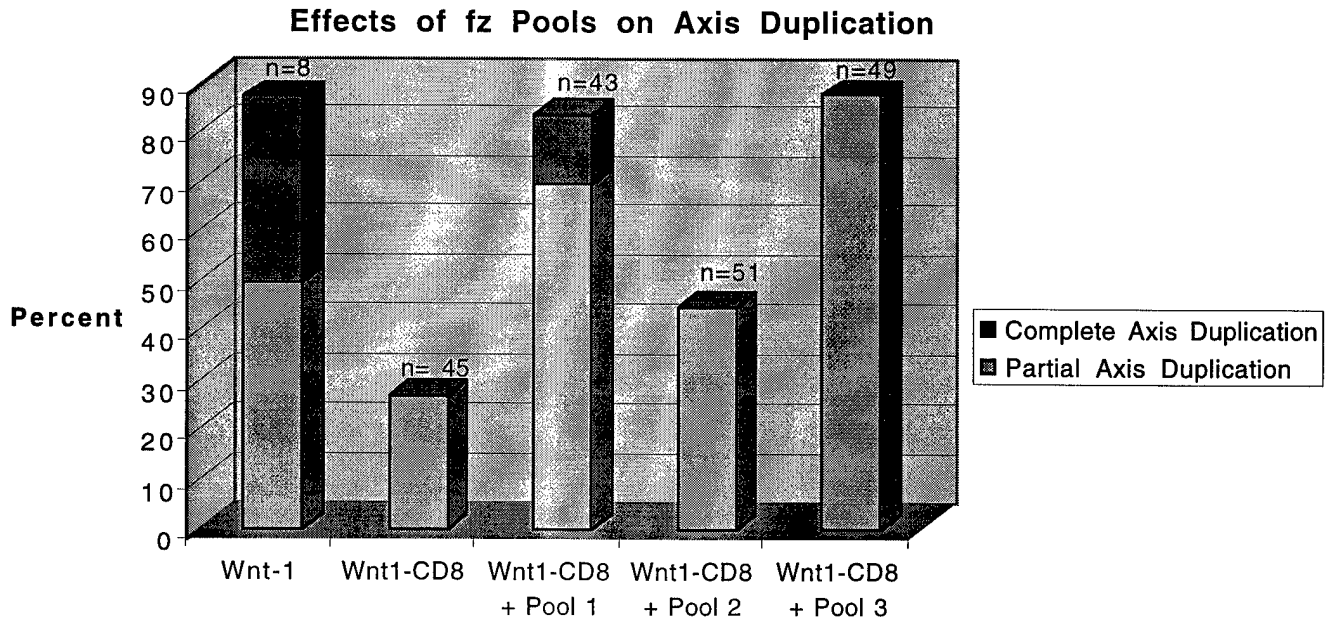


To identify any potential Wnt-1 receptor(s), ten identified mammalian *fz*-constructs and *Drosophila* dFz2 were chosen to test their ability to interact with Wnt1-CD8 construct to induce secondary axis formation. These *fz* constructs were subdivided into three pools: Pool 1 (hFz5, mFz8, Dfz2), Pool 2 (mFz3, mFz4, mFz6, FzD3 and mFzD3) and Pool 3 (rFz1, rFz2 and mFz7). Co-injection experiments of these pools of mRNA and 10 pg of Wnt1-CD8 was then performed in *Xenopus* embryos. The total amount of RNA for each *fz* construct in the individual pools was calculated to be 400 pg. The amount of injected RNA (1.2- 2.0 ng/embryo) injected is calculated to be within 20% of the maximum level (10 ng/embryo), which is considered the maximum

amount of RNA the embryo can be injected without non-specific toxic effects. It is known that the *fz* RNA pools do not by themselves induce secondary axis duplication (He et al., 1997). As a positive control, injection of the optimal amount of Wnt-1 (10 pg) was done to induce secondary axis duplication to test the responsiveness of the embryo.

The results of the co-injection experiments with the suboptimal Wnt1-CD8 construct and the three pools of *fz* RNA's revealed that both Pool 1 and 3 were able to increase the observed percentage of secondary axis formation (Figure 3). However a closer examination of these results revealed that the percentage of complete axis formation induced by these pools of RNA's in the presence of the Wnt1-CD8 construct was not as significant as the percentage seen with the Wnt-1 RNA alone. This raised the possibility that the induction of secondary axis may not be a good readout for identifying the interaction of the Wnt-1 ligand with the putative *fz* receptor. It is unlikely that the concentrations of the *fz* RNA's were not sufficient for the interaction with the Wnt-1 protein with its putative receptor, assuming that this was encoded by one of the *fz* molecules in the pools, as similar concentrations of the hFz5 receptor was shown to induce complete secondary axis formation in the presence of 10 pg of coinjected XWnt-5a (He et al., 1997).

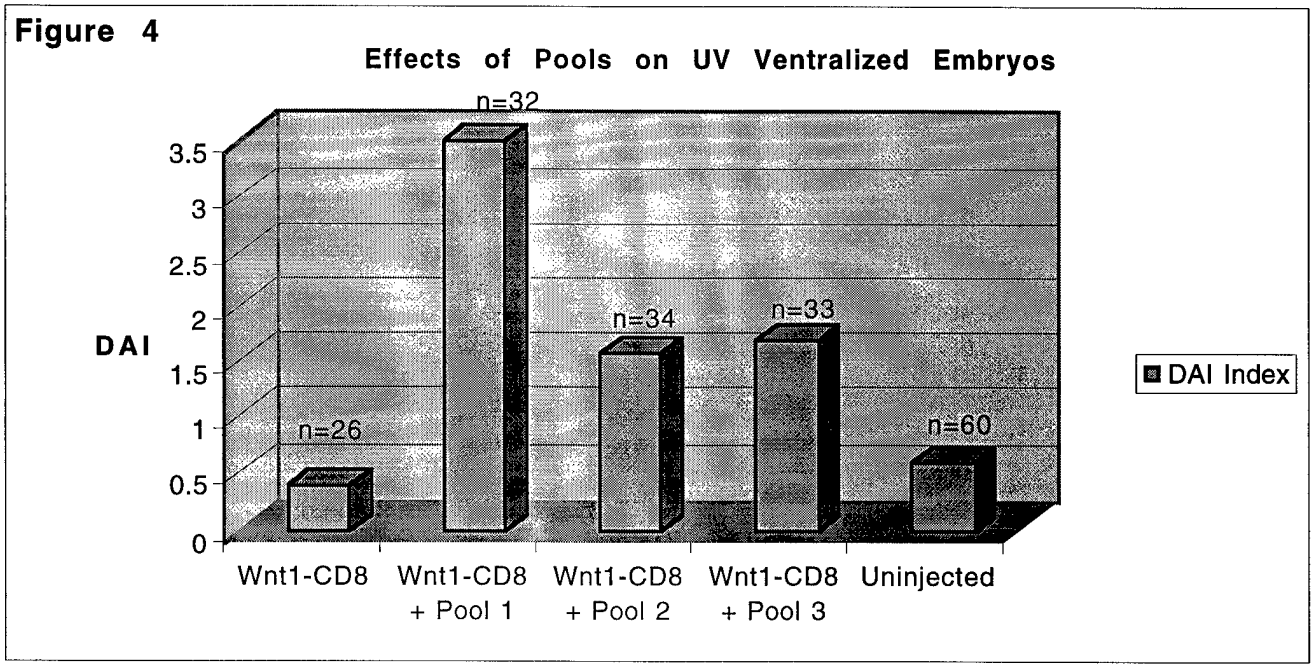
Figure 3



As such an alternative method for assessing potential interaction with Wnt1-CD8 and its potential receptor from the pools of RNA was attempted. It is known that embryos ventralized by

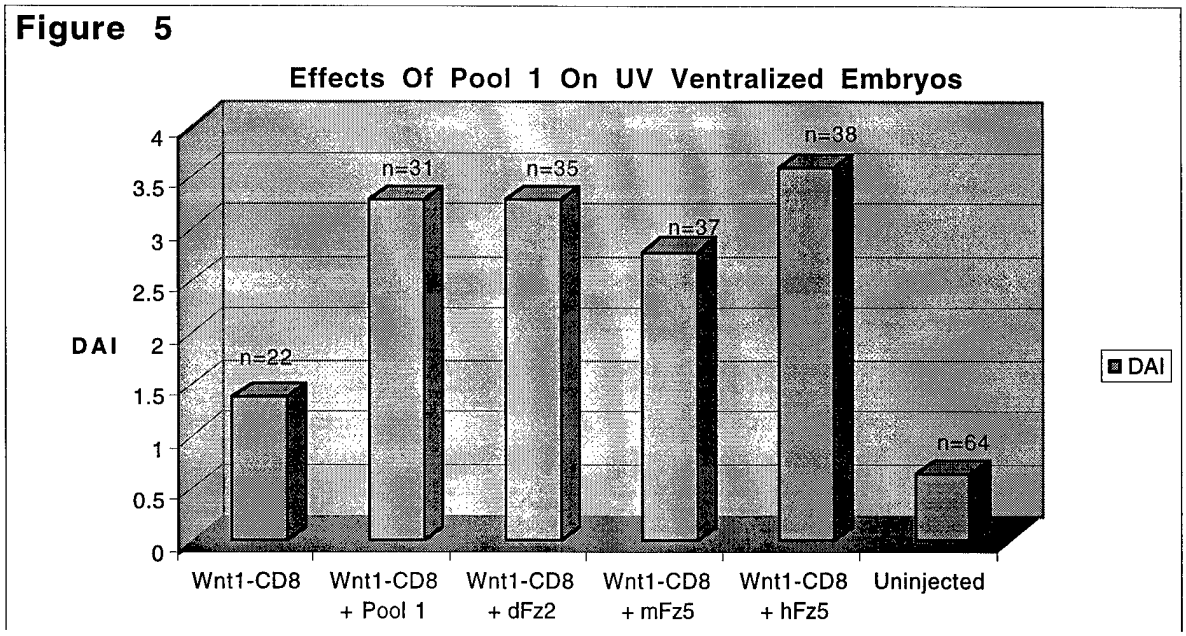
ultraviolet (UV) irradiation can be rescued by the Wg/Wnt-1 subclass of molecules (Gerhart et al., 1989). Furthermore it was shown that XWnt-5A plus hFz5 could restore dorsal development of these embryos while neither molecule by itself could accomplish this (He at al., 1997). UV ventralized embryos were then injected with the Wnt1-CD8 construct and individual pools of the *fz* RNA's and the embryos were scored by the DAI scale (Kao and Elinson, 1988) for rescue of dorsal development. As Figure 4 shows Pool 1 had the most dramatic effect in rescuing the ventralized embryos as compared to Pool 2 or 3 or Wnt1-CD8 alone.

Figure 4



This promising result prompted the investigation of which of the components of Pool 1 (hFz5, mFz8 or dFz2) was responsible for the effects seen in the previous experiment. Individual RNA's of each of the *fz* molecules (400 pg each) with Wnt1-CD8 (10 pg) were injected into UV ventralized embryos and any rescue of the ventralized phenotype was scored. As Figure 5 shows the effects of the individually injected *fz* constructs were quite similar in

Figure 5



being able to rescue the ventralized embryos. Whilst the effect of hFz5 was more pronounced than the other two molecules, this effect was not statistically significant.

Minor project not outlined in Statement of Work

A minor project that I have been addressing is directly related to the Wnt-1 signaling but not written in my submitted Statement of Work. This project involves the attempts to identify molecule(s) which interact with the carboxyl terminus of the hFz5 receptor which has been shown to induce secondary axis formation in the presence of Wnt5a (He et al., 1997). The hypothesis proposed is that as activation of the hFz5 receptor can mimic the axis induction effects seen with Wnt-1, it may directly bind to substrates involved in mediating the Wnt-1 signal transduction cascade. As such a yeast two hybrid screen has been designed with the hFz5 carboxyl terminus used the bait and fused with the Gal-4 DNA binding domain. A library containing cDNAs from mouse embryonic day 9-10, a stage when Wnt-1 signaling is known to be active will be used. As the yeast two-hybrid system can sometimes result in the identification of many positive candidate molecules, a secondary screen has been devised in which the last four amino acids (LSHV) will be deleted. These last four amino acids resemble the classical recognition site for binding of PDZ containing proteins thought to be involved in clustering or anchoring proteins on the plasma membrane. As such it is believed that removal of this sequence will provide a screen to remove any putative positive clones only involved in clustering the receptor. Furthermore a second secondary screen will be done with the carboxyl terminus of the dFz2 receptor as this receptor has the highest homology to the hFz5 receptor and is involved in mediating the Wg signal transduction cascade.

Any positives identified in this two-hybrid screen that interact with hFz5, hFz5-LSHV and dFz2 will be tested functionally for their involvement in the Wnt mediated secondary axis duplication. This can be achieved by microinjection experiments into dorsal cells of the 4-cell stage embryos and determining if the clones can directly either induce secondary axis formation or interfere with secondary axis induced by Wnt. As it is easy to test positive clones from the yeast two-hybrid screening with the established axis duplication assay in embryos, it may be possible to identify other components along the Wnt pathway that transduces the signal from the receptor. This screen can potentially lead to a better understanding of the molecular nature of Wnt signaling from the cell surface and thus lead to a better understanding of Wnt function.

Summary

The experiments performed thus far encompassed the project outlined in the Statement of Work for months 1-14. The data presented here demonstrate that the method proposed to identify the receptor for the Wnt-1 molecule contains many technical issues which have been and

continue to be addressed. While the nature of the receptor remains unelucidated, I will continue to trouble shoot the rescue of UV ventralized embryos by the individual pools of the *fz* molecules, as this technique appears to successfully distinguish effects of interaction of the Wnt-1 ligand with its putative receptor. One possibility for the outcome of the experiment presented in figure 5 is that the concentrations of the *fz* molecules may be too high and as such future experiments will address titration to lower concentrations of the components of the *fz* pools. Also it may be possible that the rescue that I am observing with Pool 1 may be nonspecific. Therefore further experiments with the individual pools of *fz* molecules will be repeated to substantiate these finding.

However the data presented that shows that two *fz* proteins may mediate the secondary axis duplication in the presence of Wnt-1 may also reflect the possibility that more than one *fz* protein may serve as a receptor for this molecule. It has been shown that the Frizzled and dFz2 proteins may serve redundant functions in mediating the effects of *wg* (Bhat, 1999 and Kennerdell and Carthew, 1998).

Continued work on identification of the Wnt-1 receptor using the *Xenopus* secondary axis duplication assay and the yeast two hybrid screen to identify proteins that interact with the hFz5 carboxyl terminus can potentially provide answers into how the Wnt-1 molecule achieves its diverse roles in mammary tumor formation and neuronal development.

Reference

1. Bhat KM. 1998. Frizzled and frizzled 2 play a partially redundant role in wingless signaling and have similar requirements to wingless in neurogenesis. Cell 95 1017-1026.
2. He X, Saint-Jeannet JP, Wang Y, Nathans J, Dawid I and Varmus HE. 1997. A member of the Frizzled protein family mediating axis induction by Wnt-5a. Science 275: 1652-1654.
3. Kao KR and Elinson RP. 1988. The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced Xenopus laevis embryos. Dev. Biol. 127: 64-74.
4. Kennerdell JR and Carthew RW. 1998. Use of dsRNA-mediated genetic interference to Demonstrate that frizzled and frizzled 2 Act in the wingless pathway. Cell 95 1017-1026.
5. Parkin N, Kitajewski J and Varmus HE. 1993. Activity of Wnt-1 as a transmembrane protein. Genes and Development. 7:2181-2193.